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Defective postnatal development of the male reproductive tract in LGR4 knockout mice

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Abstract

The final outcome of tube elongation and branching is to maximize the epithelial exchange surfaces in tubular organs. The molecular and cellular basis of these processes is actively studied in model organs such as mammary glands, liver and kidney, but they remain almost unexplored in the male reproductive tract. Here, we report that the orphan G protein-coupled receptor LGR4/GPR48 plays a role in the postnatal tissue remodeling needed for elongation and convolution of the efferent ducts and epididymis. In LGR4 knockout male mice, tube elongation fails, resulting in a hypoplastic and poorly convoluted tract. Cell proliferation is dramatically reduced in KO affected tissues, providing an explanation to the observed phenotype. Detailed analysis showed that LGR4 inactivation manifests differently in the affected organs. In efferent ducts, immune cells infiltrate the epithelium and reach the lumen, blocking the transit of sperm and testicular fluid. In addition, the hypoplasia and low convolution result in a reduction of the epithelial area involved in liquid reabsorption. Both phenomena contribute in tissue swelling upstream the blockade due to liquid and sperm accumulation, with secondary damaging effects on the germinal epithelium. In the epididymis, the thin and highly convoluted duct is replaced by a large cystic tube which is surrounded by a thick condensation of mesenchymal cells. The abnormal organization of the cellular compartments in and around the ducts suggests that LGR4 might play a role in epithelial–mesenchymal interactions. Altogether, our data identify LGR4 as an important signaling molecule implicated in the tube morphogenesis of the male reproductive tract.

Keywords: GPCR; LGR; GPR48; Male infertility; Epididymis; Efferent ducts; Tubulogenesis; Duct elongation; Proliferation; Epithelial–mesenchymal interactions

Introduction

The male reproductive system consists of a series of individual organs acting together to produce and deliver functional spermatozoa in the female reproductive tract. To be effective in fertilization, sperm cells need to mature, i.e. be capable of self-propulsion over relatively long distances and, depending on the species, they must survive during variable time in the female tract, waiting for ovulation (Jones, 1999). Once emerging from the seminiferous tubes, spermatozoa converge into the *rete testis* and exit the gonad through the efferent ducts towards the epididymis. It is mainly during their journey through efferent ducts and epididymis that sperm cells mature.

At birth, the epididymal duct is poorly developed and most of the neonatal organ volume is occupied by mesenchymal tissue. During the first days after birth, mouse epididymis undergoes extensive remodeling; the duct elongates and convolutes, extending into the space previously occupied by mesenchymal cells. However, the structure and cellular differentiation of the adult organ is only acquired during puberty (Sun and Flickinger, 1979). From birth to early adulthood, epididymal epithelial cells actively proliferate (Sun and Flickinger, 1982), contributing in the duct elongation process.

Duct elongation, convolution and branching, a process often referred to as “tubulogenesis”, are common to the development

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of many organs in the early embryo. It maximizes epithelial surfaces involved in metabolic and/or exchange functions (Hogan and Kolodziej, 2002). The cellular and molecular bases of tubulogenesis are being actively investigated in model organs like kidney, liver and mammary gland (Dressler, 2002; Lemaigre, 2003; Radisky et al., 2003); however, little is known about the mechanisms underlying tube elongation in the male reproductive tract.

Adult epididymis is a highly convoluted duct lined by a columnar epithelium, surrounded by a basement membrane and a single layer of myoepithelial cells (Robaire and Viger, 1995). It is anatomically divided into caput, corpus and cauda epididymis. These regions are functionally different: caput and corpus epididymis are implicated in maturation of sperm cells, while cauda epididymis is mainly a place of storage. In caput epididymis, at least three compartments (I, II and III) can be identified on the basis of histology, ultrastructure and gene expression. Epithelial and stereocilia height decreases from compartment I to III, and continues decreasing towards the corpus and cauda regions. Ultrastructural characteristics associated with secretory and absorptive functions also allow to distinguish the epithelial cells from the different compartments (Abe et al., 1983). This regionalization is also physically defined by the existence of connective tissue septa that, in addition to their structural support role, seem to precisely delimit areas characterized by the expression of specific genes (Turner et al., 2003; Luedtke et al., 2000).

Circulating androgens are needed for development of epididymis and efferent ducts, from the beginning of sexual differentiation up to the end of puberty. Several lines of evidence have demonstrated that fetal sexual differentiation and early postnatal development are regulated by testosterone which is produced independently of gonadotrophin activity; on the contrary, the morphological modifications of the ducts acquired during puberty are regulated by the gonadotrophin-dependent testosterone increase (Lei et al., 2003; Zhang et al., 2003, 2004).

In addition to circulating androgens, lumicrine factors produced by the testis are also involved in epididymis physiology. When lumicrine signaling is blocked by ligation of the efferent ducts, epididymal gene expression is disrupted (Chauvin and Griswold, 2004).

Knockout mice models have revealed the role of several genes in epididymal or efferent duct physiology and development. Disruption of estrogen receptor alpha (ERα) causes male infertility secondary to a defect in reabsorption of testicular liquid by efferent ducts (Hess et al., 1997). BMP4, 7, 8a or 8b inactivation results in epithelial degeneration of precise areas of the epididymis, indicating a region-specific requirement of different classes of BMPs for epididymal epithelium functioning (Chen et al., 1999; Hu et al., 2004; Zhao et al., 1998, 2001). Another animal model that contributed to the understanding of epididymis development is the c-Ros knockout. Inactivation of this tyrosine kinase receptor results in male sterility due to agenesis of the initial segment of caput epididymis (Sonnenberg-Riethmacher et al., 1996).

In the present study, we report the implication of the gene encoding the G protein-coupled receptor LGR4/GPR48 (hereafter called LGR4), in the postnatal development of the male reproductive tract. LGR4 belongs to the LGR (Leucine-rich G protein-coupled Receptors) subgroup of the rhodopsin-like GPCR superfamily (Hsu et al., 2000). They are characterized by a large extracellular domain, with multiple leucine-rich repeats, involved in receptor–ligand interaction (Kobe and Deisenhofer, 1994). From a phylogenetic point of view, vertebrate LGRs can be classified in three groups: the FSH, LH and TSH receptors or GpHr (for Glycoprotein Hormone Receptors, Vassart et al., 2004); the relaxin receptors group, which includes the recently deorphanized LGR7 and LGR8 (Hsu et al., 2002); and the orphan LGR group which includes LGR4, LGR5 and LGR6 (Hsu et al., 1998; Loh et al., 2001; Hermey et al., 1999; Hsu et al., 2002).

It was reported recently that LGR4 knockout (LGR4KO) mice show in utero growth retardation and neonatal lethality, preventing analysis of the postnatal and adult phenotype (Mazerbourg et al., 2004). Here, we show that mice harboring the same LGR4 mutation on a different genetic background are viable and most of them reach adulthood. LGR4KO males are infertile due to abnormal postnatal development of the reproductive tract, involving efferent ducts and epididymis.

Materials and methods

Animals

Animals were housed in a temperature (21 ± 1°C) and humidity (55 ± 10%)-controlled room with a 12 h light:12 h dark cycle (light between 7.00 A.M. and 7.00 P.M.). Food and water were available ad libitum. Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the Local Ethical Committee.

LGR4 mutant mice

LGR4/GPR48 mutant mice were generated in a gene-trap screen described previously by Leighton et al. (2001). The mutant allele name has been designated as Gpr48^Gt(pGT0)1Ah. The secretory trap vector pGT0TMpfs is inserted in intron 1 of LGR4/Gpr48 to produce a transcript containing exon 1 (that codes for the first 62 amino acids containing the signal peptide and cysteine-rich-N-terminal region) fused to the bgeo-PLAP cassette. Heterozygous Gpr48^Gt males (initially in a 129Ola × C57Bl/6 hybrid background) were outcrossed 6 generations to CD1 animals. The homozygous Gpr48^Gt mice are referred to as LGR4KO throughout the text.

Inverse PCR

Genomic DNA prepared from tail biopsies were digested with *Sau*3A I restriction enzyme. The generated fragments of 200–400 bp were circularized with T4 DNA ligase (Gibco BRL). Insertion region was amplified from the circular DNA mix by PCR, using a couple of divergent primers 86r (CACTCCAACCTCCGCAAACTC) and 89f (TGTTGTAGGACACGAACCCCAG). PCR product was sequenced using primer 86r.

RNA-based genotyping

We set up a genotyping assay based on the analysis of LGR4 mRNA phenotype. We generated cDNA from 1 μg of total RNA purified from tail biopsies with two allele-specific primers, one that anneals on LGR4 wild type mRNA (*wtRT*: GGCGACCAGGAAAATGAACCACACTGT) and the second that anneals on the vector cassete (*mut RT*: GCGGATCATCGGTCAGACGATTCAT) (Supplementary Fig. 1A). 1/10 of each retro-transcription product was used as template in the PCR reaction.

The forward primer is common to the mutated and the WT products and anneals on LGR4 exon1 (*fwd*: ATGCCGGGACCGCTGCGGCTGCTCT). Reverse primers anneal on LGR4 exon 5 (*wt rev*: GACGCACAGGCACTTCCGTCAAGAT) or on the trap-vector sequence (*mut rev*: GGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAA). PCR was performed in standard conditions for 35 cycles and 55°C as annealing temperature.

Tissue preparation for histology

Organs were fixed by intracardiac perfusion of 4% paraformaldehyde (PAF) in PBS pH 7.4. Testis, epididymis and efferent ducts were dissected out at different postnatal (P) ages. Collected tissues were postfixed for additional 12–20 h in PAF 4%, dehydrated, embedded in paraffin and then sectioned at 8 μm. In most cases, the whole testis or epididymis was sectioned and representative samples throughout the organ were mounted on slides (SuperfrostPlus) for hematoxylin and eosin, or immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was carried out according to standard procedures. The following primary antibodies were used: rabbit anti-rat NHE3, and rabbit anti-rat AQP1 (dilution 1/1000, Chemicon), mouse anti-human ER-α (dilution 1/100, Novocastra), rat anti-mouse CD45/LCA (dilution 1/25, BD Pharmingen). Appropriate secondary antibodies were used according to provider instructions (Chemicon, BD Pharmingen).

Whole-mount LacZ staining

Efferent ducts were dissected in PBS, digested with collagenase to remove connective tissue, fixed in acetone for 30 min at 4°C (to dissolve lipids), then rehydrated for 15 min in PBS at 4°C. Samples were then washed in a LacZ-buffer (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS) for 30 min at 4°C (protected from light) and then incubated 30 min at 37°C in LacZ-buffer containing 0.5 mg/ml X-gal. Reaction was stopped by washing in PBS and postfixing in PAF 4%.

In situ hybridization

Wild type adult CD1 mice were fixed by intracardiac perfusion with PAF 4%. Organs were dissected and refixed overnight at 4°C. Tissues were cryo-sectioned and submitted to in situ hybridization according to standard procedures. The RNA digoxigenin-labeled probes consist of the sense and antisense mRNA corresponding to the complete extracellular domain of LGR4. Negative controls were done with the corresponding sense probe. The hybridized RNAs were evidenced by immunodetection of digoxigenin with an antidigoxigenin antibody coupled with alkaline phosphatase (Roche).

BrdU incorporation

BrdU (50 μg/g of body weight) was administered intraperitoneally to P3 WT and KO mice. Mice were sacrificed 1 h later, perfused with PAF 4% and postfixed for 24 h. Following histological processing, paraffin sections were rehydrated, denatured 30 min in 2 M HCl and neutralized in sodium borate 15 min (0.1 M pH 8.5). BrdU incorporation into DNA was revealed with a mouse anti-BrdU antibody (dilution 1/50; BD Pharmingen) followed by secondary donkey anti-mouse antibody coupled to Cy3 (Chemicon). Epithelial and mesenchymal cell compartments were analyzed in 100× magnification fields. Values were obtained by counting 8 to 12 100× fields on 4 to 6 sections of each WT and KO organ. Results are expressed as percentage of labeled cells.

Trypan blue staining

We set up a protocol to visualize the epithelial absorption and the integrity of the tract in efferent ducts. Trypan blue is a dye known to stain absorptive epithelia as the proximal tubule of the kidney (Zemanová et al., 2002). 500 μl of trypan blue 0.5% was subcutaneously injected two times, 3 days spaced, to 4 week-old WT and KO mice. Animals were killed 1 week after the first injection, efferent ducts were dissected out and immediately fixed in PAF 4%. The whole tissue was observed under a LSM510 NLO confocal microscope fitted on an Axiovert M200 inverted microscope equipped with a C-Apochromat 40×/1.2 N.A. water immersion objective (Zeiss). The 543 nm excitation wavelength of the HeNe1 laser, a main dichroic HFT 488/543/633 and a long-pass emission filter (LP560 nm) were used for selective detection of the red fluorescence emission of trypan blue in efferent duct cytological environment. Afterwards, the efferent ducts were processed for histological examination as described above.

Results

Insertion site of the gene-trap vector

The LGR4/GPR48 mutant allele [Gpr48^Gt(pGT0)1Ah] was generated in a gene-trap screen of mutations in secreted and membrane-spanning proteins for those that perturb brain wiring in mice (Leighton et al., 2001). To set up a genotyping assay, we determined the precise integration site of the gene-trap vector by inverse PCR (see Materials and methods). It is inserted 5932 nucleotides downstream from the 5′ end of intron 1 (nucleotide position 50756422 ref|NT_039207.3|Mm2_39247_33) (see on-line Supplementary Fig. 1A, Mutant allele).

Integration of the mutagenic cassette in the LGR4 gene generates a null allele

The efficacy of the gene-trap mutagenic strategy depends on correct mRNA processing of the mutagenic cassette in the context of the trapped gene. For the generation of a null allele, the SV40 polyadenylation signal of the vector must be fully functional and the cassette must be recognized by the splicing machinery as a constitutive exon to be joined to upstream transcribed sequences (Supplementary Fig. 1A; for vector details, see Leighton et al., 2001). In order to address whether the mutagenic cassette of the trap vector inserted in LGR4 intron 1 is efficiently processed to generate a null allele, we systematically genotyped the animals by analyzing the LGR4 mRNA phenotype in tail biopsies. The LGR4 gene has been shown to be strongly expressed in the cartilage of intervertebral discs (Van Schoore et al., 2005).

Three clear band patterns were obtained which correspond to the expected homozygous wild type (+/+ or WT), heterozygous (+/− or HE) and homozygous mutant (−/− or LGR4KO) patterns (Supplementary Fig. 1B). The WT transcript is not detected in the homozygous mutants, demonstrating that the mutagenic cassette is correctly processed and the trapped gene effectively generates a null allele.

No in utero or perinatal mortality in −/− LGR4 knockouts

We analyzed the genotypic frequencies in 12 litters. From 125 newborns genotyped at postnatal day 7 (P7), 46% were

HE, 27% were WT and 27% KO. These values correspond to the expected mendelian frequencies, indicating that there is no in utero or perinatal death associated to LGR4 mutation on the CD1 genetic background. However, we observed that the health of some KO individuals progressively deteriorates during the period of milk feeding, leading to early death of these animals.

LGR4KO are smaller than wild type littermates

At postnatal day 7 (P7), LGR4KO are 28 and 25% smaller than WT and HE littermates, respectively (Fig. 1A). Animals were also weighted at 14, 21, 28, 35, 42 and 49 days postpartum, showing that LGR4KO keep their weight deficit at all ages and reach a body weight plateau earlier than WT and HE littermates (Fig. 1B).

To search for gross abnormalities in organogenesis, LGR4KO organs were weighted and the results expressed as a fraction of body weight. A disproportionate reduction was only observed for testis/epididymis (data not shown).

The male reproductive tract in LGR4KO

Each KO male was caged with two WT females during 3 to 5 weeks. Copulatory plugs were observed indicating that LGR4KO males mate normally but no pregnancies were obtained. No gross external anomaly was observed in LGR4KO males for testis descent, ano-genital distance and morphology of papilla (not shown). Upon dissection, *vas deferens*, seminal vesicles and prostate looked anatomically and histologically normal (not shown). In contrast, several features of testis, efferent ducts and epididymis were abnormal in all animals (Figs. 2A and B): (i) a bilateral edema, sometimes associated with the presence of red cells, was observed in the *rete* region of the testis, (ii) efferent ducts and epididymis were severely hypoplastic and (iii) the epididymal fat was reduced (not shown).

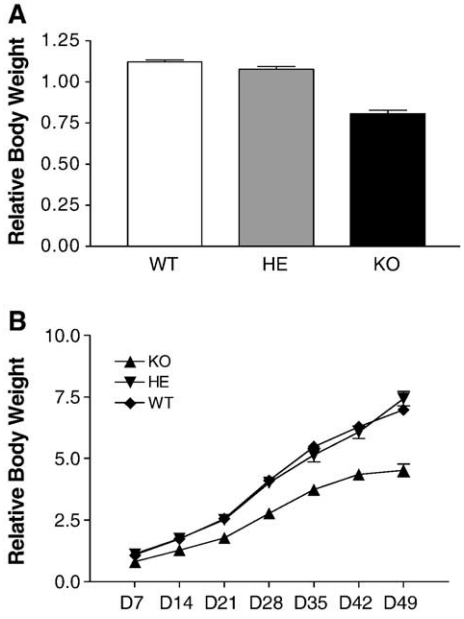

Fig. 1. Mice body weights and growing curve. (A) Pups were weighted at P7 and classified according to their genotypes. In order to group mice of the same genotype, but coming from litters which varied in the mean pup size (due to the natural variability in the outbred genetic background), weights were expressed as a ratio between each individual weight (iw) and the mean weight of the corresponding litter at D7 (mw) iw/mw = Relative Body Weights. (B) Growing curve. Mice were weighted at postnatal days 7, 14, 21, 28, 35, 42 and 49. The Relative Body Weights were calculated and classified by genotypes. The mean values of each genotype were graphed along the time. Results are expressed as mean ± SEM.

Testis

In histological preparations, we observed a dilation of the *rete testis* where large amounts of sperm cells and liquid accumulate. In extreme cases, almost half of a longitudinal section was occupied by the dilated *rete testis* (Fig. 2F). Signs of germinal epithelium atrophy, as thinning of the germinal cell layer and the consequent increase in the lumen size, were observed in the seminiferous tubes (Figs. 2G and H). The presence of spermatozoa in the seminiferous tubes and *rete testis*, and of Leydig cells in the interstitial space (Figs. 2G and H, arrowheads), is an indication of normal spermatogenesis and testicular function; though, they may be progressively disrupted as the phenotype develops (see below). The dilation of the *rete testis* region, the enlarged lumen of seminiferous tubules and the atrophy of the germinal epithelium are only observed after the onset of puberty (Fig. 2D). Before that period, testicular tissue looked normal (not shown).

Efferent ducts

Efferent ducts structure was analyzed in whole-mount and histological preparations. Remarkably, the duct bundle is less convoluted and hypoplastic in LGR4KO (Figs. 3A and B). At the proximal side (close to the testis), ducts are dilated and their lumina are filled up with sperm cells. Immune cell granulomas are found close to the dilated ducts in the surrounding tissue (Figs. 3C and D and insets). The hypoplasia and low convolution of the ducts, as evidenced by the number of duct cross-sections, are already observed at the onset of puberty in three weeks-old animals (Figs. 3G and H and insets). At this age, the duct diameter is also drastically reduced (Figs. 3G and H).

In adult mice, certain epithelial characteristics as cell height and pseudo-stratification show strong variation between tubes (Figs. 3E and F, upper panel). Accordingly, three different regions can be identified at the proximal side of the efferent ducts of LGR4KO: (1) regions with a very flattened and non-pseudostratified epithelium, where the ciliated cells are not present; (2) regions with a flattened and non-pseudostratified epithelium but with ciliated cells in which the nuclei are located basally; (3) regions in which the epithelium height and pseudostratification are nearly normal (Figs. 3E and F, upper panel).

In contrast to the proximal side, at the distal region of efferent ducts (close to the epididymis), the epithelial

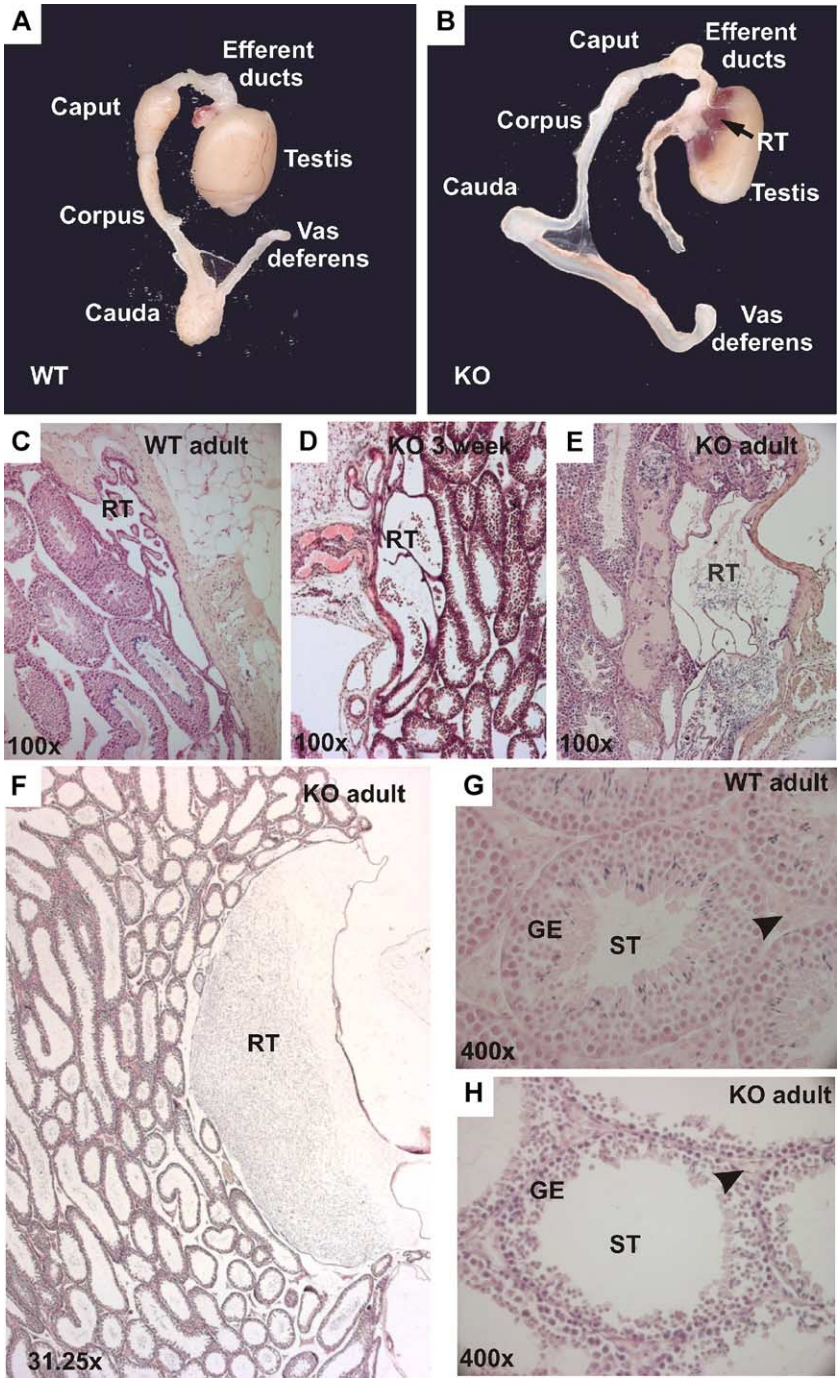

Fig. 2. Liquid and sperm cells accumulation in the testis. (A and B) Testis, efferent ducts and epididymis after dissection of the epididymal fat. The *rete testis* (RT) in LGR4KO is shown (black arrow). (C and F) Representative hematoxyline–eosin histological preparations at the *rete* area of the testis. Normal *rete testis* in adult WT mice (C). RT dilation in P23 LGR4KO (D). In adults, RT continues dilating and fills with spermatozoids (E). Low magnification photograph of an adult testis where the dilated RT extends into the germinal tissue (F). (G and H) Representative histology of the seminiferous tubes (ST). Adult ST of WT (G) and KO (H) adult mice. The luminal enlargement of ST and thinning of the germinal epithelium (GE) are shown. Arrowheads indicate the presence of Leydig cells.

height, the presence of ciliated cells and the pseudostratified aspect were similar to WT (Figs. 3E and F, lower panel).

We thus conclude that disruption of the epithelial cytoarchitecture at the proximal side is a consequence of local liquid and sperm accumulation and the associated immune reaction.

Epididymis

The epididymis is macroscopically abnormal (Figs. 2A and B). The epididymal fat is reduced (not shown) and a thinner, underdeveloped structure substitutes the typical inflated normal caput epididymis. In histological preparations, we observed that the characteristic highly convoluted and narrow tract is replaced by a dilated, much less convoluted duct,

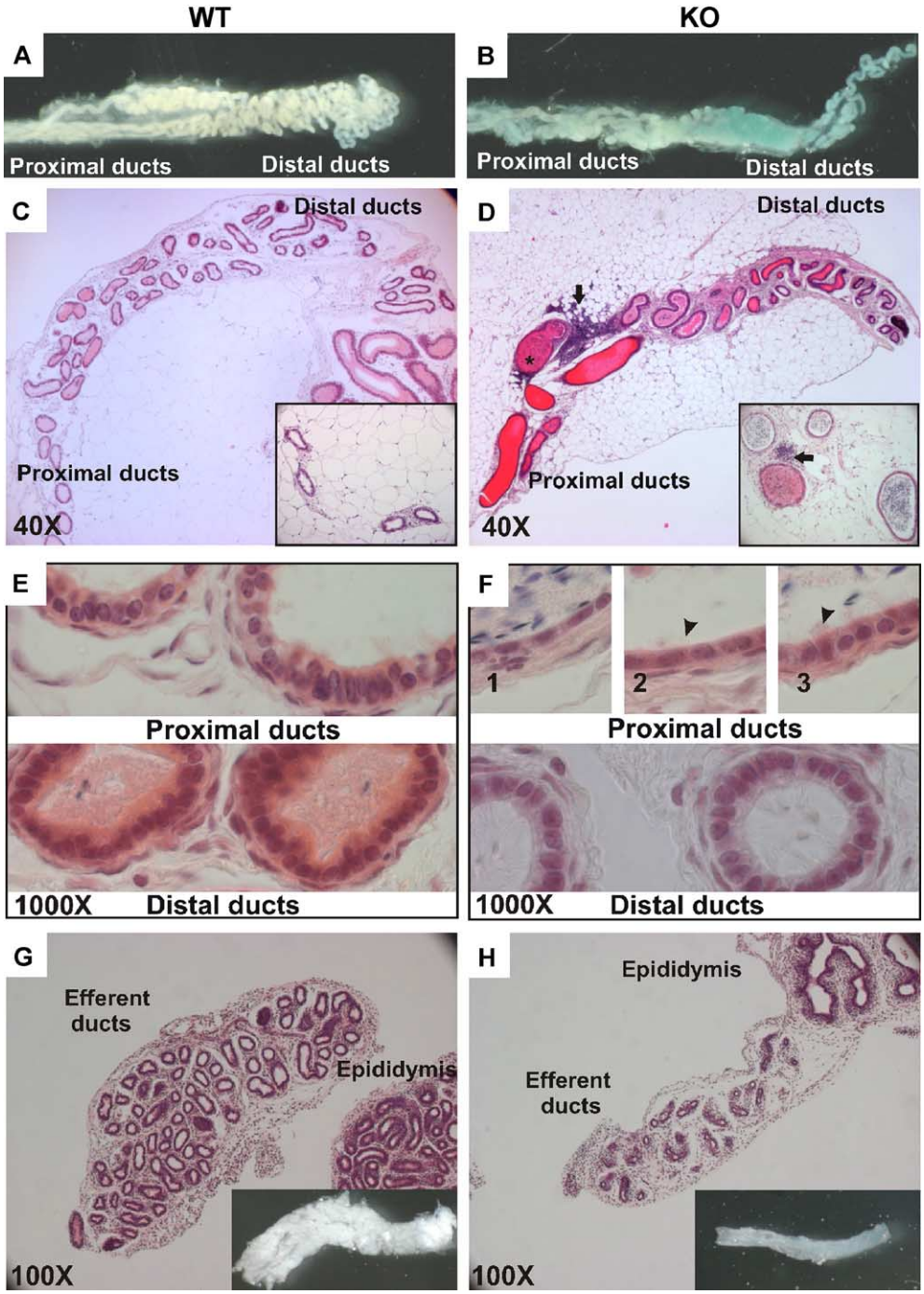

Fig. 3. Morphological defects in efferent ducts. (A–F) Adult efferent ducts. Whole-mount preparations of WT (A) and KO (B) efferent ducts stained using beta galactosidase activity encoded in the mutagenic cassette inserted in LGR4 mutant allele. Longitudinal and transversal (insets) sections of WT (C) and KO (D) organs showing the dilation, the sperm cell accumulation (*) and the immune cell granuloma at the proximal region (arrows). High magnification images of the proximal (upper panel) and distal (lower panel) efferent ducts of WT (E) and KO (F) mice show the epithelial structure at the proximal region. Longitudinal sections and whole-mount preparations (insets) of WT (G) and KO (H) 3 weeks-old efferent ducts highlighting the hypotrophy and low convolution in LGR4KO.

which is surrounded by a thick condensation of mesenchymal cells (Fig. 4, adult).

In WT adult epididymis, the epithelium height is maximal at the initial segment and decreases gradually through the caput, towards the corpus. This cell shape pattern is absent and the epithelial cell height is almost constant throughout the caput and corpus regions of LGR4KO. The average height of the epithelium in caput region is reduced by 60% as compared to WT (18.34 ± 4.58 μm vs. 43.94 ± 9.78 μm).

In this flattened epithelium, a bilayer of nuclei is observed in most of caput and corpus cross-sections (Fig. 4, adult, inset a). Additionally, epithelial buds protrude from the principal duct all along the epididymis (Fig. 4, adult, inset b).

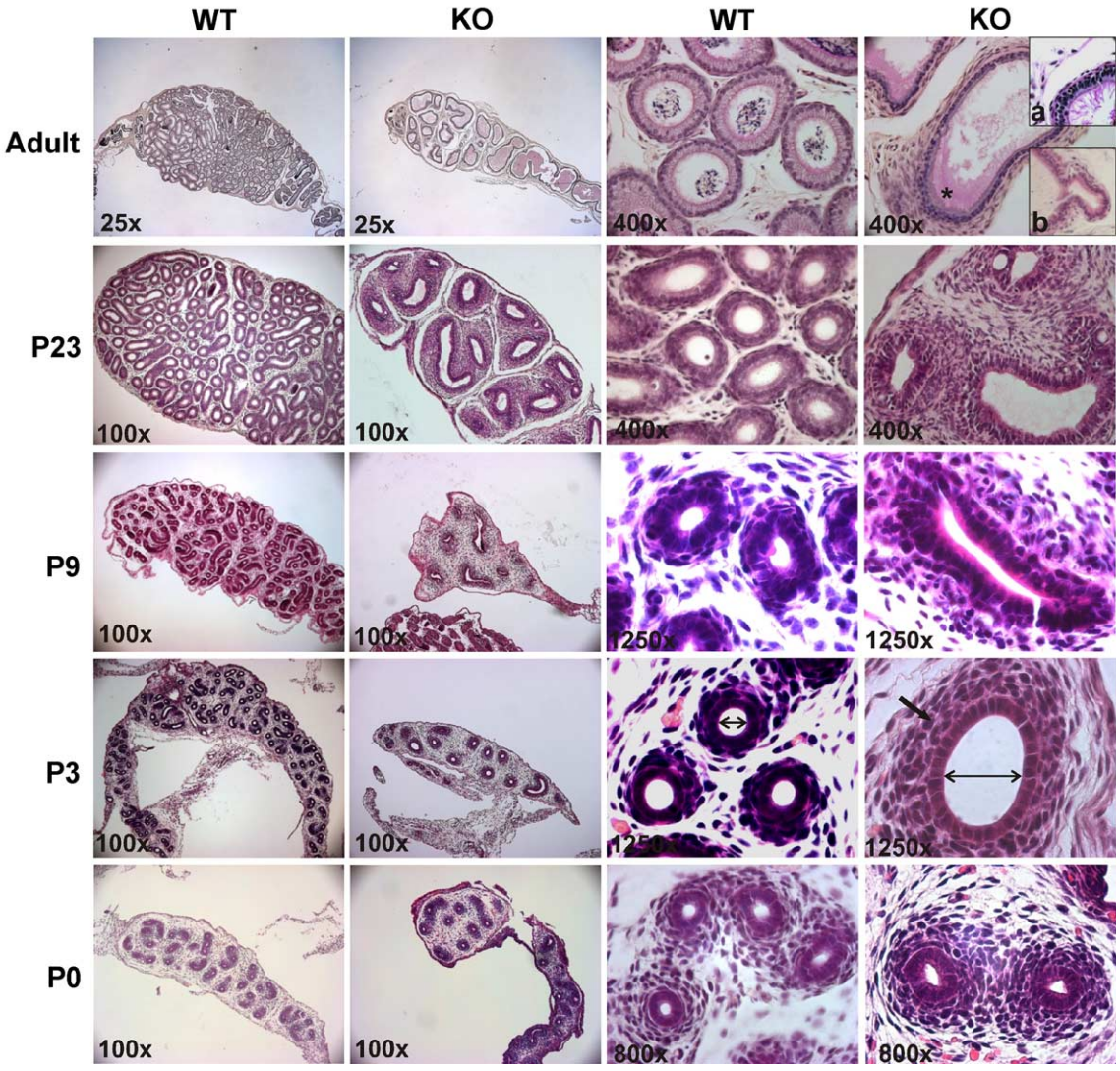

Fig. 4. Postnatal development of the epididymis. Longitudinal sections of caput epididymis stained with hematoxylin–eosin to show the organ structure and the evolution of the phenotype from birth to adulthood. Higher magnification images (the two columns at the right side) illustrate the lumen dilation (double-headed arrows, P3) and the mesenchymal cell condensation (black arrow, P3) around the duct. An eosinophilic secretion (*) fills the lumen of the adult duct. (a) Higher magnification image showing two layers of nuclei of the LGR4KO epithelium. (b) Epithelial buds projecting from the epididymal duct.

In most animals, epididymis was completely devoid of sperm cells.

The epididymal phenotype is established during early postnatal development

We studied the evolution of the epididymal phenotype from birth to puberty. At birth, WT and LGR4KO epididymis look similar (Fig. 4, P0). However, in 3 day-old animals, the overall organ structure as well as the duct histology are already abnormal. The diameter of the duct is enlarged, the number of cross-sections is severely reduced in KO (Fig. 4, P3), and the tube is surrounded by a thicker condensation of mesenchymal cells. These features indicate a defect in elongation of the tube during the first days of the postnatal development, which evolves towards a cystic structure and does not catch up during subsequent days and puberty (Fig. 4, P9 and P23).

Reduced cell proliferation in efferent ducts and caput epididymis

To determine whether the defect in duct elongation is associated with a reduction of cell proliferation, we performed BrdU incorporation experiments in P3 mice. The number of labeled cells is markedly reduced in the epithelium of caput epididymis and efferent ducts of LGR4KO (Fig. 5). It is also decreased, though less markedly, in the mesenchymal compartment of caput epididymis, but not of efferent ducts. There was no significant difference in apoptotic cell number, assessed by the TUNEL reaction, between WT and LGR4KO during postnatal development until adulthood (not shown).

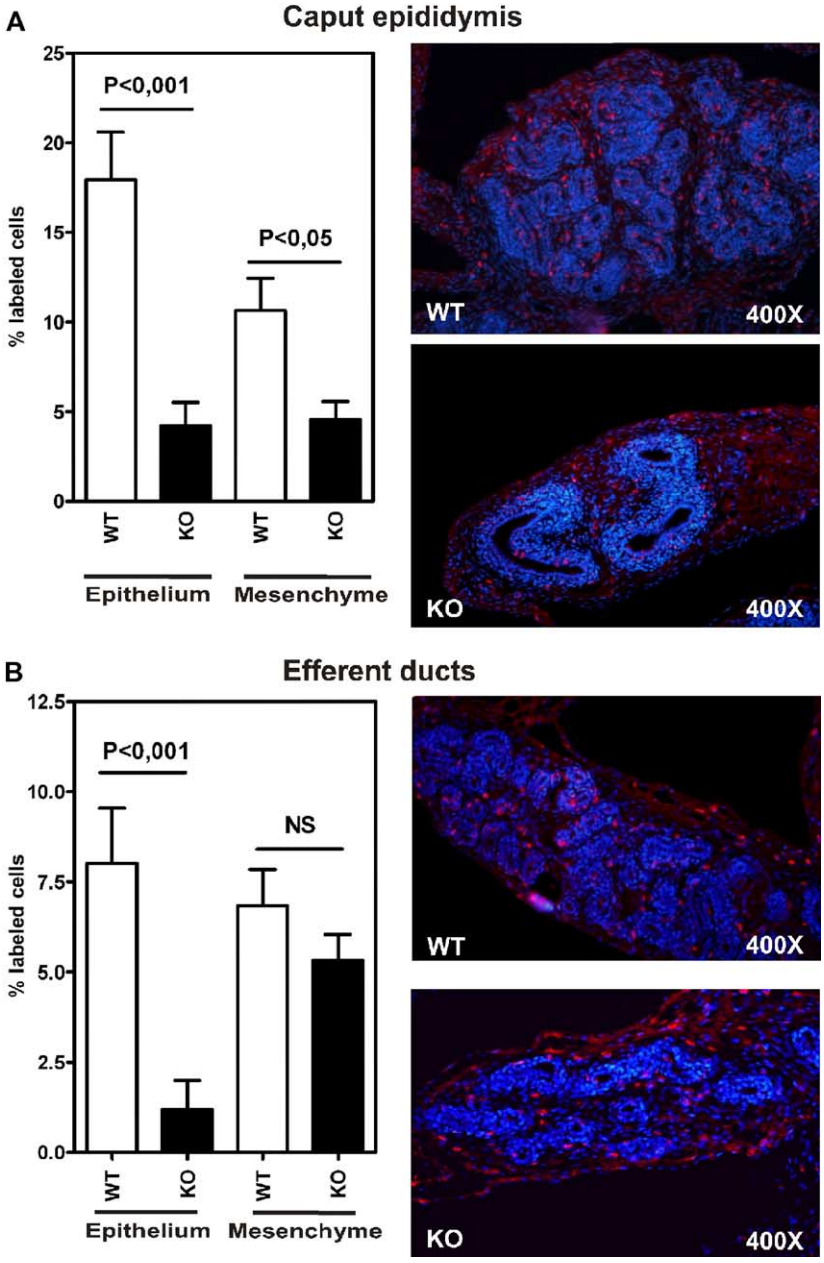

Fig. 5. Cell proliferation in epididymis and efferent ducts. Proliferating cells were labeled by BrdU incorporation into their DNA. Epithelial and mesenchymal-labeled cells (red nuclei) were counted in caput epididymis (A) and efferent ducts (B). Results were expressed as percentage of labeled cells (red nuclei) over total cells (blue nuclei). White and black bars represent the calculated percentages in WT and KO tissues, respectively. Values represent the mean ± SEM of 8 to 12 quadrants according to groups. Statistical significance was determined by Student's *t* test. N.S.: non-significative.

LGR4 is expressed in the testis, efferent ducts and epididymis throughout males postnatal development

We studied LGR4 expression in the testis, efferent ducts and epididymis by in situ hybridization on wild type adult tissues. The mRNA is detected in the seminiferous tubes, with higher staining intensity at the basal side of the germinal epithelium, and in the epithelium lining the epididymis and the efferent ducts (Figs. 6A–F). In the epididymis, the signal is stronger at region I (initial segment) of caput and decreases gradually towards regions II, III and corpus (Fig. 6C). LGR4 mRNA is not detected in cauda region (not shown).

We studied the expression of LGR4 during development of the male reproductive tract, by measuring activity of the beta-geo reporter gene in heterozygous mice. LGR4 is expressed in testis, efferent ducts and epididymis at all stages tested and, in particular, all along the period during which the phenotype of LGR4KO is established (P0, P3 and P9, Figs. 6G–L).

Expression of estrogen receptor α (ERα), Na^+/H^+ transporter 3 (NHE3) and Aquaporin 1 (AQP1) in LGR4KO efferent ducts

ERα, NHE3 and AQP1 are all expressed in the epithelial cells of efferent ducts. Interestingly, the three corresponding

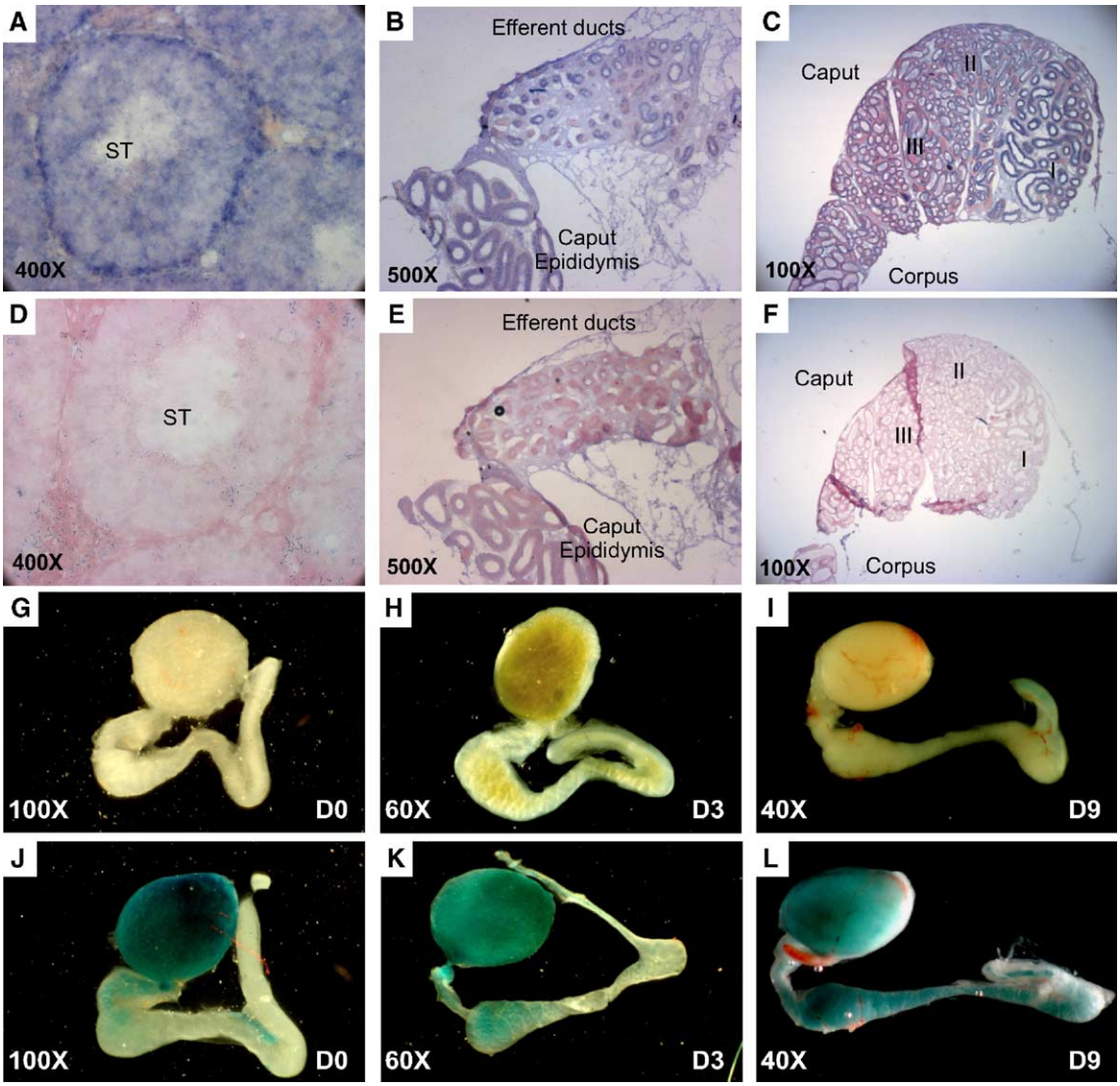

Fig. 6. Expression of LGR4 mRNA in male reproductive tract. (A–C) Expression of LGR4 mRNA studied by in situ hybridization on adult WT tissues hybridized with the antisense RNA probe; (D–F) tissues hybridized with the sense RNA probe. ST: seminiferous tubes. I, II and III: compartments of caput epididymis. Corpus and cauda regions hybridized with the sense probe are not shown. (G–L) Staining of whole-mount genital tract of adult heterozygous (J–L) or wild type male mice (G–I) at day D0, D3, D9 and showing expression of the beta-geo reporter gene encoded in the gene-trap cassette.

male knockout mice share the same phenotype (Zhou et al., 2001): a dilation of *rete testis* and efferent ducts due to stasis of testicular secretion, which was explained by a defect of liquid reabsorption.

On the basis of the similarities between these knockout models and LGR4KO, we studied the expression of ERα, NHE3 and AQP1 along LGR4KO efferent ducts. The localization and intensity of the immunostaining of the three proteins were normal in most of the efferent ducts regions analyzed (Fig. 7, columns at the left). However, in the proximal segment, the staining of the three proteins was reduced or undetectable (Fig. 7, columns at the right), in the same areas where spermatozoa and testicular fluid accumulate and the epithelial cytoarchitecture is disrupted (see Figs. 3E and F). This suggests that expression of ERα, NHE3 and AQP1 is an additional epithelial characteristic which is disrupted as a consequence of the sperm accumulation and the immune reaction at the proximal side of efferent ducts.

The transit of sperm and testicular fluid through efferent ducts is physically blocked in LGR4KO

To further explore the mechanisms responsible for the accumulation of sperm cells and testicular secretion in the efferent ducts and testis, we reasoned that staining the lumen of efferent ducts with a dye may allow to visualize the integrity of the circuit. Trypan blue is a vital dye that has been previously used to evaluate the absorption capacity of certain epithelia (Zemanová et al., 2002). When injected subcutaneously, the dye enters the blood stream, passes the blood-testicular barrier, dilutes in testicular secretions and reaches the efferent ducts where it is absorbed and concentrated by the epithelial cells. Uptake of the dye by epithelial cells may give us an idea about

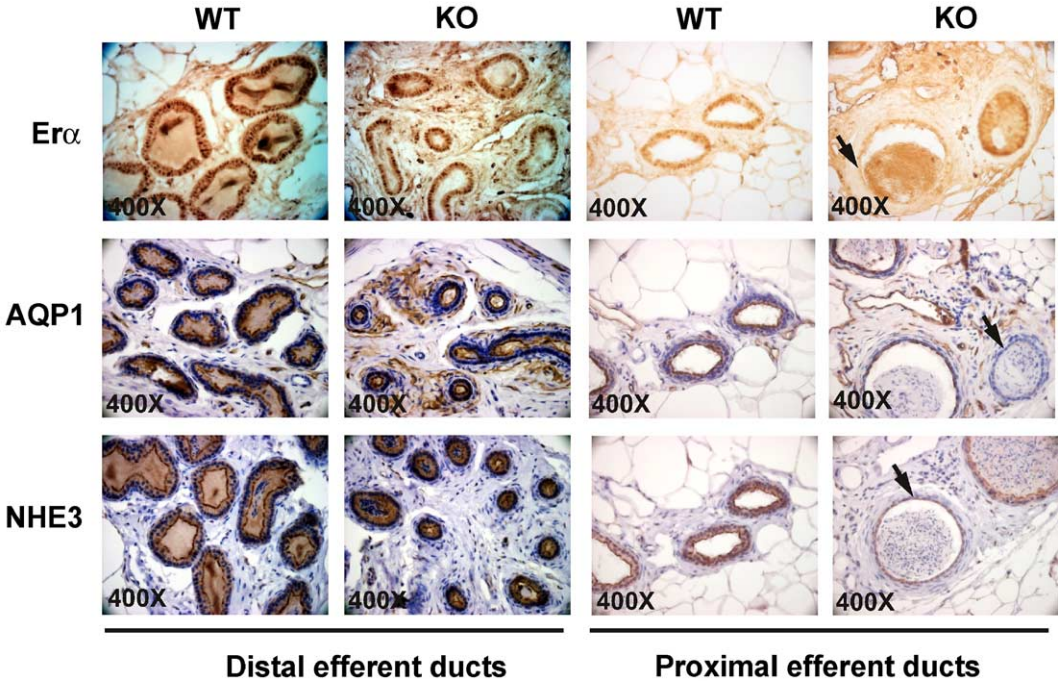

Fig. 7. ERα, NHE3 and AQP1 immunoexpression and localization in LGR4KO. Expression of ERα, NHE3 and AQP1 proteins was studied by indirect immunohistochemistry on WT and LGR4KO adult proximal and distal efferent ducts. Arrows indicate the duct cross-sections where the immunoexpression is abnormal at the proximal side of efferent ducts.

the integrity of the efferent ducts circuit and, at the same time, give clues about the absorptive capacity of the epithelium. Four week-old mice were injected with trypan blue, and efferent ducts were examined 7 days later in whole-mount preparations by fluorescent confocal microscopy. At this age, spermatozoa have not yet emerged from seminiferous tubes, but testicular liquid is already actively secreted.

Efferent ducts from WT and LGR4 KO take up the dye efficiently, as evidenced by the red fluorescence concentrated in epithelial cells (Figs. 8A and B). However, in LGR4KO, absorption of the dye stops abruptly nearby the cone region (Fig. 8B, arrow). Inspection of histological sections of this region shows that the lumen is filled up with cellular material (Fig. 8B, insets). Part of the cells present in the lumen of the efferent duct expresses CD45 (also known as Leukocyte Common Antigen), a protein that is present in all leukocytes, thus indicating their immune origin (Fig. 8F). In adult mice, also next to the region where sperm cells stop flowing, we found several syncytial-like structures at the basal side of the epithelium pushing the epithelial cell layer towards the luminal space (Figs. 8C and F). At some places, they deform the lumen of duct (Figs. 8C and D), at others, they break the epithelial wall and invade the lumen (Fig. 8E). These syncytial-like structures located at the base of the efferent duct epithelium were also labeled with the anti CD45 antibody (Figs. 8G and H), providing evidence of their immunological origin.

Discussion

In our attempt to identify the functions and agonists of the LGR rhodopsin-like subfamily of GPCRs, we and others reasoned that important clues could be provided by the study of knockout mice of the corresponding genes (Mazerbourg et al., 2004; Morita et al., 2004). An LGR4KO mouse line was generated in the frame of a gene-trap experiment aiming at genes involved in axon guidance (Leighton et al., 2001). Whereas the marker proteins encoded in the gene-trap vector allowed the construction of a detailed atlas of LGR4 expression sites (Mazerbourg et al., 2004; Van Schoore et al., 2005), the function of the receptor remained unexplored. Mazerbourg et al. reported that LGR4KO animals displayed intrauterine growth retardation with no obvious developmental defects, and die around birth of undefined cause.

In the present study, we showed that LGR4KO mice are viable. When litter genotypes were assessed 1 week after birth, segregation of the mutated allele showed no deviation from the expected mendelian frequency, indicating that LGR4KO do not die in utero or around birth. The use of the CD1 outbred strain to backcross the LGR4 mutant allele, in contrast to the inbred C57bl6 strain used by Mazerbourg et al., is most probably the factor favoring mice survival in our experiments.

We confirm that newborns are smaller than wild type and heterozygous littermates, and show that this difference in body size is maintained throughout life. Even though the majority of LGR4KO reaches adulthood, the health of some individuals deteriorates progressively during the milk feeding period leading to early death. One possible explanation is a disadvantage of LGR4KO in competition for food related to their reduced body size.

The survival of LGR4KO allowed us to further study the phenotype resulting from LGR4 loss of function. KO males

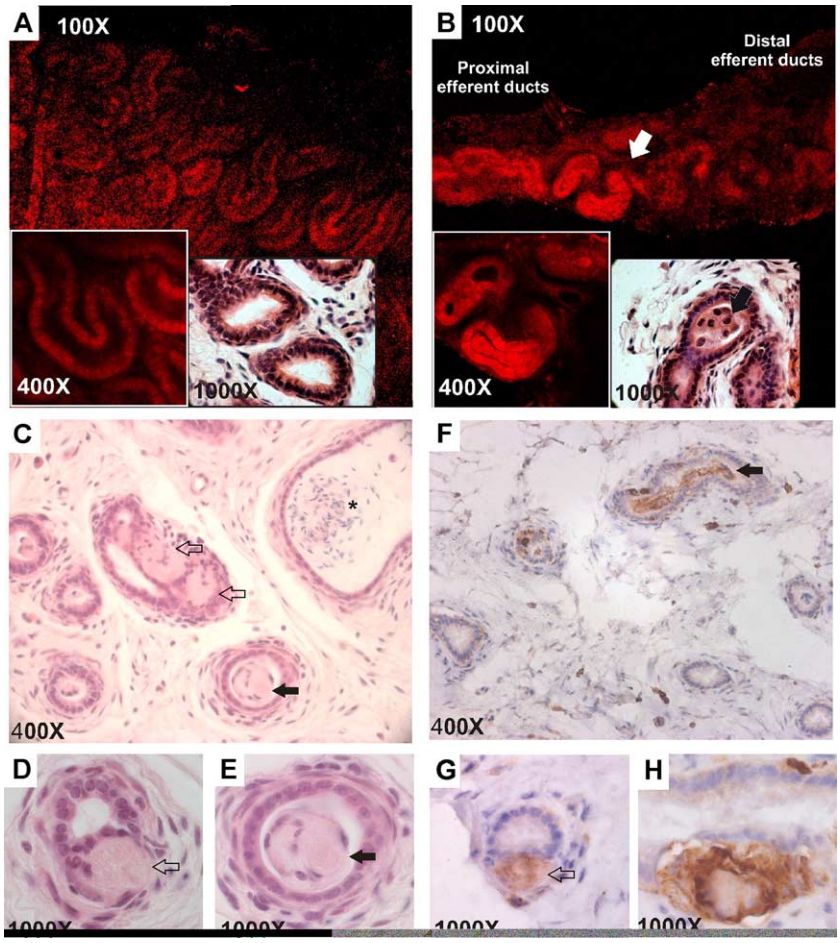

Fig. 8. Fluid circulation blockade at the efferent ducts. Whole-mount efferent duct preparation of WT and KO mice injected with trypan-blue observed under the fluorescent confocal microscope. The dye concentrates in epithelial cells and emits red fluorescence when illuminated with long pass band centered on 543 nm. (A–B) Low and high (inset) magnification of WT (A) and LGR4KO (B) efferent ducts. The region where the dye absorption and the fluid circulation stop are shown (white arrow and dark field inset). The same tissue was longitudinally sectioned and stained with hematoxylin and eosin to characterize the luminal content (Bright field insets). (C–E) Sections of adult efferent ducts showing the syncytia-like structures located at the basal side of the epithelium and inside the lumen (empty and black arrows, respectively). These structures are usually found immediately downstream the region where sperm cell accumulate (*). (F–H) Immunolabeling of adult efferent duct with an antibody directed against the LCA (leukocyte common antigen) or CD45 used to distinguish leukocytes from non-hematopoietic cells. The content of the lumen is clearly labeled in certain cross-sections (F, black arrow) while it is negative in others. (G–H) The syncytia-like structures located at the base of the epithelium are also labeled by the anti CD45 antibody.

are sterile and display a malformation of the reproductive tract. Upon dissection we found: (1) liquid accumulation in the *rete* region of the testis, (2) severe hypoplasia of efferent ducts and epididymis, (3) normal *vas deferens*, seminal glands and prostate (not shown), (4) accumulation of sperm cells in *rete testis* and the proximal portion of the efferent ducts, (5) immune cells infiltrating the lumina of the efferent ducts and the surrounding mesenchyme, (6) a dramatic enlargement of the lumen of the epididymal duct and reduction in its length and convolution, (7) an abnormally thick condensation of mesenchymal cells around the epididymal duct and (8) absence of sperm cells in the epididymis of most of the analyzed animals.

The initial suggestion that the overall duct dilation (in *rete testis*, efferent ducts and epididymis) could be simply produced by a blockade of sperm and testicular secretions downstream of caput epididymis does not fit with the content of the ducts: sperm is present in efferent ducts, but virtually absent in epididymis.

By analogy with other KO models, we considered that the dilation of the *rete testis* could be explained by a progressive increase in the luminal pressure due to defective reabsorption of the testicular fluid. This reabsorption process, which is important to concentrate sperm at the outlet of the testis, is mainly accomplished by the efferent ducts (Ilio and Hess, 1994). Interestingly, ERα, NHE3 and AQP1 knockout mice display *rete testis* and efferent ducts dilation due to liquid accumulation during the period coinciding with the onset of testicular secretory activity (Zhou et al., 2001). In ERαKO, it has been shown that fluid reabsorption in the efferent ducts is impaired (Hess et al., 1997) as a consequence of downreglation of NHE3, a protein with known function in ion transit through epithelia. It was therefore proposed that estrogens, via ERα, controls liquid absorption by regulating the expression of key genes in the efferent ducts (Zhou et al., 2001).

In LGR4KO mice, *rete testis* dilation and liquid accumulation are also observed at the onset of testicular fluid secretion,

suggesting that a similar process might be involved. However, the normal expression of NHE3, AQP1 and ERα in LGR4KO efferent ducts argues against this hypothesis. An interesting particularity of LGR4KO is a defect in the postnatal development of efferent ducts and epididymis that results in a severe hypoplasia of both structures. One functional consequence in the efferent ducts is the reduction of epithelial surfaces available for fluid exchange, expected to negatively affect fluid reabsorption capacity. Our observations therefore support the hypothesis that a decrease of liquid absorption could also be a factor implicated in the phenotype of LGR4KO, but with a mechanism different than the one proposed for ERα, NHE3 and AQP1 knockouts. Whereas in ERαKO, the primary defect consists of inadequate regulation of genes (like NHE3 or AQP1) directly involved in fluid/ion transport through the epithelium of the efferent duct, a developmental defect leading to abnormal organogenesis and consequent reduction of the absorptive surface would be one of the factors contributing to liquid accumulation in LGR4KO. In any case, the finding that testicular fluid and spermatozoa flow through efferent ducts is physically blocked indicates that an additional factor is playing in LGR4KO. The blockade is mainly made of immune cells that infiltrate the efferent duct epithelium and lumen. The mechanism leading to this infiltration within and around the lumen of the efferent ducts is not immediately apparent. We initially thought that it could be due to breakage of the epithelial walls, secondary to dilation of the ducts by accumulation of sperm cells and testicular secretion. However, immune cells infiltrate the epithelium and the lumen of efferent ducts of 4 to 5 week-old animals, before the arrival of spermatozoa. We are thus left with the hypothesis that the developmental anomaly affecting the efferent ducts is accompanied by defective establishment of the barrier function played by the epithelium. This would allow immune cells, normally intercalated in the epithelium (Robaire and Hermo, 1988), to invade the lumina of the ducts interfering with the normal flow of testicular fluid.

During early postnatal development, the normal epididymis undergoes an extensive tissue remodeling which results in elongation and convolution of the duct. Two weeks after birth, the structure of the adult organ is in place. However, final differentiation of the epididymal epithelium is only achieved after a second wave of transformations which occurs at the onset of puberty, induced by the increase of gonadotrophin-dependent androgens and testicular secretions. LGR4KO display a clear defect of the early postnatal phase of tissue remodeling. Whereas at birth the epididymis seems anatomically and histologically normal, at P3 duct elongation fails, with a concomitant reduction of BrdU incorporation which is particularly marked in the epithelial compartment (Fig. 5). The result is striking in caput epididymis which does not acquire its normal convoluted aspect and displays a very enlarged lumen, evoking evolution towards cystic structures. This growth deficit at the moment of active tissue remodeling might explain the severe hypoplasia observed in both efferent ducts and epididymis. Whether LGR4 is directly involved in controlling cell proliferation, or acts indirectly, will only become clear when its agonist is available for experimentation.

In addition to cell proliferation, correct directional cell division and/or intercalation are required for the elongation of any duct system (Germino, 2005). Defects at this level are now proposed as general mechanisms leading to cyst formation, with cystic disease of the kidney providing a much investigated example (Kierszenbaum, 2004; Kuo-Jung et al., 2005). The overall cystic aspect of the LGR4KO epididymis, together with the fact that the epididymis and the renal tubules derive from the same embryonic structure (the Wolffian duct), suggests the possibility of a shared pathogenetic mechanisms with cystic disease of the kidney. Against this hypothesis, however, we found no gross anomaly in the kidney of young LGR4KO, nor in other Wolffian-derived organs as *vas deferens* and seminal glands.

The dilated epididymal duct in LGR4KO is surrounded by an abnormally thick condensation of mesenchymal cells. A similar condensation was described in caput epididymis of the PDGF-A KO mice. As PDGF-A and PDGF receptor α localize in epithelial and mesenchymal compartments, respectively, a paracrine control of epididymal morphogenesis was suggested (Basciani et al., 2004). Most of the available information about the mechanisms and genes involved in growth of urogenital tubes comes from the study of the kidney development. For example, HGF, TGF-β and BMP4, all expressed in the metanephric mesenchyme, promote elongation of the ureteric bud, in vitro and/or in vivo, demonstrating the importance of mesenchymal signaling in this process (Miyazaki et al., 2000; Bush et al., 2004; Davies, 2001; Stuart et al., 2001). Recently, the role of epithelium-mesenchyme interactions has been highlighted to understand the basis of tubulogenesis (Hogan and Kolodziej, 2002). In the situation described here, the abnormal arrangement of epithelial and mesenchymal compartments, together with the defect in tube elongation, both probably associated with (or secondary to) the reduction in epididymal cell proliferation, suggests that interactions between epithelium and mesenchyme may occupy a central role in establishment of the LGR4KO phenotype. However, until progresses are made about the nature and cellular origin of LGR4 agonist, we can only speculate about a paracrine control of epididymis development involving LGR4 during the early postnatal period.

In addition to the duct elongation defect evidenced soon after birth, the epididymal epithelium of LGR4KO also fails to acquire the normal differentiated state of the adult, during the second developmental wave occurring at puberty (Fig. 4). This anomaly takes place despite a series of indicators of normal androgen activity during development and adulthood (testis descent, differentiation of external sexual organs, sexual behavior, serum testosterone levels, the presence of normal amounts of Leydig cells; data not shown), thus arguing against a role of circulating androgens in the phenotype.

Blocking the transit of testicular secretions by ligation of efferent ducts is known to prevent adult differentiation of the epididymal epithelium and development of the initial segment of caput epididymis (Abe et al., 1984a,b; Avram and Cooper, 2004), as well as normal protein expression (Hermo et al.,

2000), emphasizing the role of lumicrine factors present in the testicular secretions in epididymal physiology. Interestingly, mice in which the c-Ros tyrosine kinase receptor has been inactivated also fail to develop the initial segment of caput epididymis (Sonnenberg-Riethmacher et al., 1996). Based on this similarity, it was suggested that the unknown c-Ros ligand might be a lumicrine factor present in the testicular fluid (Avram and Cooper, 2004).

In LGR4KO, the blockade in fluid transit at the level of efferent duct could account for the absence of adult differentiation of epididymal epithelium. However, it is unlikely to explain the epididymal cystic dilation and the defect in duct elongation, which establish 2 weeks before the beginning of testicular secretory activity. When efferent ducts are ligated at birth in WT animals (Abe et al., 1984a,b), the initial segment of the epididymis fails to develop but the overall structure of the duct is far from resembling the epididymis described here. This suggests that the early developmental phenotype of LGR4KO is independent of postnatal lumicrine factor(s) coming from the testis.

In summary, our data indicate a role for LGR4 in tube morphogenesis of the efferent ducts and the epididymis, probably via a positive effect on epithelial and mesenchymal cell proliferation. We propose that defective tubulogenesis results in a decrease of fluid/ions absorption secondary to a reduction in the total epithelial surface in the efferent ducts of LGR4KO. In addition, the epithelium fails to exert its barrier role that prevent the contact between the immune system and spermatozoa, allowing the infiltration of immune cells that subsequently occlude the ducts. Both phenomena contribute to liquid and sperm accumulation and swelling of proximal efferent ducts and testis. In the epididymis of LGR4KO, the duct fails to elongate making a large cystic tube, surrounded by a thick condensation of mesenchymal cells, in place of the thin and convoluted normal tract. The abnormal cell arrangement found in and around the ducts suggests that LGR4 could play a role in epithelial–mesenchymal interactions. Our findings identify LGR4, and its still unknown agonist, as important players in the development of the male reproductive tract, and qualify LGR4KO as a new model for the study of tube morphogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.11.043.

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